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PROGNOSTIC EVALUATION OF ANGIOGENESIS AND LYMPHANGIOGENESIS USING ENDOTHELIAL MARKERS IN BREAST CANCER

MA. ANSARI[1], V. SRIVASTAV[2], M SAHOO[3], M. KUMAR[4] & A. KUMAR[5]

[1]Associate Professor, Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

[2]Assistant Professor, Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

[3]Post-Graduate Resident, Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

[4]Professor, Department of Pathology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

[5]Professor, Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

ABSTRACT

Controversy exists regarding relationship between angiogenesis and lymphangiogenesis, and their usefulness as prognostic factor in breast cancer. So for a quantitative assessment of angiogenesis and lymphangiogenesis, we determined microvessel density (MVD) and lymphatic vessel density (LVD) in 40 invasive breast cancer patients, by using specific endothelial markers like CD31 and D2-40 respectively with Weidner's immunohistochemistry technique. Then statistically correlated them with each other and among other well-known poor prognostic factors like tumor size, stage, grade, axillary lymph node status, vascular invasion and hormone receptor status. We found a strong correlation between MVD and LVD ($p < .001$) and also a significant association of both MVD and LVD independently with tumor size ($p=.003$; $p=.007$), stage ($p= .021$; $p=.046$), metastatic lymph nodes ($p=.004$; $p=.041$) and lymphatic vessel invasion (LVI) seen on H&E staining ($p= .016$; $p=.022$) but not with LVI seen on D2-40($p=.016$; $p=.242$). With grade, although LVD show a significant association ($p= .028$), MVD shows insignificant association ($p= 0.136$). However no association exists with total lymph nodes yield. So with this valuable results we may conclude that angiogenesis and lymphangiogenesis are closely related and are poor prognostic factors in breast cancer.

KEYWORDS: Cancer Breast, Angiogenesis, Lymphangiogenesis

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in female worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008[1]. The incidence of breast cancer is higher in developed countries, and in developing countries it is the second highest cause of death in women after cervical cancer[2]. A current report says, by the year 2020, breast cancer is set to overtake cervical cancer as the most common type of cancer among all women in India[3].

So proper prognostication is mandatory for prevention and management of primary breast cancer. Prognostication, however, is a multivariable process, as the outcome of a disease is determined by a variety of (sometimes interacting) factors. In this respect, several prognostic indicators has already been established, including age, tumor size, axillary lymph node status, histological grade, tumor type, vascular invasion, and hormones receptor status [4,5]. These prognostic factors allow a better comprehension of the natural history of the disease and the identification of homogeneous populations of patients with a similar outcome profile. Recent developments in cytogenetic and molecular biology have provided new ways to assess prognosis [6].

Studies over various tumors have shown that angiogenesis (blood vessel growth) and lymphangiogenesis (lymphatic growth) correlate significantly with tumour growth, invasion and metastasis[7] and they may share some common regulatory features. Thus microvessel density (MVD) and lymphatic vessel density (LVD) as a measurement for angiogenesis and lymphangiogenesis respectively may be considered as reliable markers predictive of tumour aggression and metastasis. The most useful pathological approach for assessing angiogenesis and lymphangiogenesis involves microscopic estimation of blood or lymphatic vessel density on tissues probed for endothelial markers by immunohisto chemistry (Weidner's or Chalkey's method). Several endothelial markers have been developed but at present pan-endothelial markers like anti CD31, CD34, and CD105 are used routinely for IHC assessment of angiogenesis. Similarly anti D2-40 is most commonly used endothelial marker for assessment of lymphangiogenesis. Apart from prognostic importance, these markers might be useful for therapeutic decision making in early cancers. However, the College of American Pathologists has stated that further study of quantification of tumour angiogenesis and lymphangiogenesis is still required to demonstrate its prognostic value in breast. Since there role as prognostic factors has not been firmly established[5].

Thus the aim of this study were to (1) prognostic evaluation of angiogenesis and lymphangiogenesis by estimating microvessel density (MVD) and lymphatic vessel density (LVD) by immunohistochemistry using CD31 and D2-40 antibody as an angiogenetic and lymphatic endothelial marker respectively. (2) Then to correlate MVD and LVD with established clinicopathological prognostic parameters in breast cancer viz. tumor size, lymph node status, grade, stage of disease, hormonal receptor status, Her2/neu status.

MATERIALS AND METHODS

Patients and Specimen

This was a prospective study conducted at department of General Surgery in collaboration with department of Pathology, Institute of Medical Sciences, Banaras Hindu University, from July 2011 to May 2013. Forty consecutive female patients with early invasive ductal breast carcinoma without neo-adjuvant chemotherapy or hormone therapy or previous local surgical intervention were included in this study. All patients were admitted after proper informed and written consent. A detailed history of disease course, parity, family history and clinical evaluation was done to determine tumour size, infiltration, axillary lymph node status and stage of the disease. Two out of 40 patients underwent breast conservative surgery and in the rest modified radical mastectomy was done as primary treatment modality. The surgical specimen was sent for detailed histopathological evaluation which includes: type, tumor size, pathological stage, grade (Nottingham grading system), total lymph node yield, total positive lymph nodes with metastasis, lymphatic and vascular invasion using H&E staining. After histopathological examination, the formalin fixed paraffin embedded tumour blocks were sent for immunohistochemistry which includes determination of hormone receptor status also.

Assessment of MVD and LVD

Blocks of the viable tumor representative area were selected for immuno-histochemistry (IHC). Tissue sections (4 µm) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6), using microwave method. 2 cycle of 10 min each first at 95^0C and second at 97^0C. Then after cooling slides were washed in TRIS buffers for 5 min in 3% hydrogen peroxide to quench endogenous tissueperoxidase. Primary monoclonal antibodies were directed against CD31 (1:80dilution, "BIOGENEX", Netherlands) and D2-40 antigen (1:50dilution, "LABVISION", USA). Again washing with TRIS buffer for 3 minutes, sections were covered with HRP labeled secondary antibody solution and incubated in humid chamber for 30 minutes at room temperature. Then staining was done by immersing slides in 0.05%3, 3' Diaminobenzidinetetrahydrochloride. Thereafter all slides were counterstained with hematoxyline, dehydrated and mounted.

Weidner's criteria (1991) were used for assessment of MVD and LVD. The sections were initially scanned at low magnification (10x), thereby finding area with the highest number of microvessel in all the fields of each slide at the periphery of the tumour (hot spot). Three hotspots were selected and counting of all CD31 and D240 stained vessels lumen were done on separately stained slides under high magnification (40 x). Counting was performed by two independent observers.

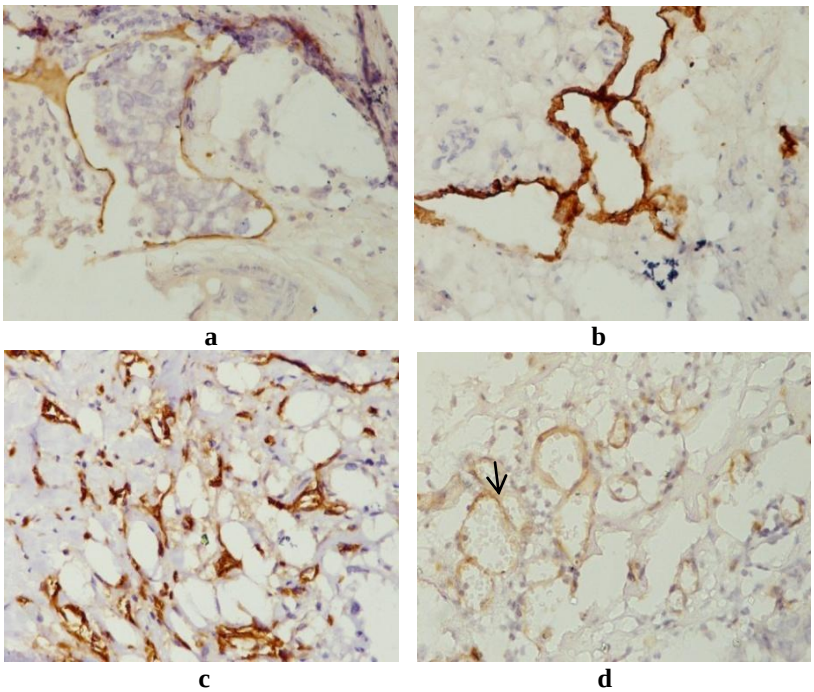

Figure 1: (a), (b) Example of D2-40 Facilitates Lymphatic Tumor Invasion Detection D2-40 (40x Magnification) Lymph Vessels Containing Tumor Cells (Indicated by Arrow) (c), Example of CD31 Stained Slides (40x Magnification) (d) A CD31 Stained Slide at 40X Magnification where There is Tumour Emboli in Blood Vessel (Indicated by Arrow)- A Rare Phenomenon

Within the same immunostained slides any vascular or lymphatic embolies were marked if found, which shows presence of MVI or LVI. Almost all invasions noted were lymphatic. Vascular invasion were rarely encountered.

STATISTICAL ANALYSIS

Three levels of statistics were performed using the SPSS version 16:

(a) Pearson's correlations (b) 2X2 chi squaretest and (c) Student T- tests. Pearson's linear correlation was used to show correlation among clinicopathological factors with numeric values like BVD, LVD, tumor sizes, total lymph node count and total positive lymph node count. Chi sq. test use to find association of factors with ordinal variables (i.e. high. intermediate and low) like stage and grade with BVD and LVD. Student T test was used for finding correlation between factors with binary nominal values (present/absent) like LVI, hormone receptor status with other factors. Statistical analysis was 2- tailed and significant was defined if P value <0.05.

OBSERVATION

Patient's demographic and clinic opatho logical profile is illustrated in table 1.

The mean micro vessel density (MVD) was 11.45 ± 3.94 micro vessels per x40 field and mean lymphatic vessel density (LVD) was 17.92 ± 7.38 lymphatic micro vessels per x40 field visually. Based on the mean, patients were classified into low and high MVD and LVD. Table 2, shows Pearson's correlations between micro vessel density MVD and lymphatic vessel density LVD and with other prognostic factors with numeric values. Both show a strong positive correlation between each other, where linear Pearson's correlation coefficient (r) =0.545 and a statistically significant P value < 0.001(2-tailed). Also MVD and LVD individually shows a significant correlation with tumor size (r = 0.454, p= 0.003; r = 0.422, p= 0.007) and total positive lymph nodes (r= 0.447, p=0.004; r=0.321. p=0.041). Whereas, no correlation exist with total lymph nodes count (r=0.251, p=0.118; r= - 0.007, p=0.966). Using chi^2 test, we found high MVD shows a statistically significant association with stage and insignificant association with grade of the tumor (table 3).Similarly, high LVD shows significant association with higher stage (χ^2=3.956; p=0.046) and also with grade (χ^2=4.800; p= 0.028).When student T- tests was used, LVI seen with H&E stain show significant positive correlation with both MVD and LVD. Whereas, LVI detected with D2-40, found to be insignificantly correlated to both LVD and BVD. Estrogen negative receptor status, correlate significantly with MVD only. How ever, insignificant correlation with PR, Her2neu status. LVD however did not show any significant correlation with any hormone receptor status.

Table 1: Clinicopathological Profile

Clinicopathological Parameters	f (n=40)	% (n=100%)	Clinicopathological Parameters	f (n=40)	% (n=100%)
Age (Years)			**Tumor Size (cm)**		
< 40	12	30	<5	26	65
≥ 40	28	70	≥ 5	14	35
Duration (Months)			**Stage**		
< 6	14	35	Early invasive IIa	9	22.5
6 - 12	23	57.5	IIb	17	42.5
> 12	3	7.5	Locally advanced IIIa	14	35
Presenting Symptom/s			**Grade**		
Breast lump	40	100	Low I + II	30	75
Axillary lump	5	12.5	High III	10	25
Nipple discharge	5	12.5	**TLN**		
Pain	13	32.5	0	2	5
Parity			**1 - 3**	**2**	**5**
Nulliparous	6	15	4 - 9	13	32.5
single child	2	5	> 10	23	57.5
Two children	13	32.5	**PLN**		
Three children	19	47.5	0	11	27.5
Menstrual Status			**1 - 3**	**12**	**30**
Premenopausal	17	42.5	4 - 9	11	27.5

Table 1: Contd.,

Postmenopausal	23	57.5	> 10	6	15
Family History (+/-)	1/39	2.5/97.5	**LVI(H&E)+/-**	16/24	40/60
Breast Feeding (+/-)	34/6	85/15	**LVI(D2-40)+/-**	26/14	65/35
Tumour Site (Left/Right)	**22/18**	**55/45**	**Hormone Receptor Status**		
Nipple Involvement (+/-)	5/35	12.5/87.5	ER (+/-)	15/25	37.5/62.5
Skin Fixity (+/-)	7/35	17.5/82.5	PR (+/-)	24/16	60/40
Palpable Axillary LN (+/-)	30/10	75/25	Her2neu (+/-)	9/31	22.5/77.5

"+/_" denotes present /absent

Table 2: Pearson's Linear Correlation between MVD and LVD with Each Other and Other Prognostic Parameter

		MVD	LVD
MVD	Pearson r	1	.545**
	p-val ue		<0.001
LVD	Pearson r	.545**	1
	p-value	<0.001	
Tumor size	Pearson r	.454**	.422**
	p-value	0.003	0.007
TLN	Pearson r	0.251	-0.007
	p-value	0.118	0.966
PLN	Pearson r	.447**	0.321
	p-value	0.004	.041*

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 3: Comparison between MVD and LVD with Stage and Grade

Stage/Grade	MVD				LVD			
	Low (<11.45)		High (>11.45)		Low (<17.92)		High (>17.92)	
	No.	%	No.	%	No.	%	No.	%
Stage								
Early invasive (IIa +IIb)	19	79.16	7	43.75	16	80	10	50
Locally advanced (IIIa)	5	20.84	9	56.25	4	20	10	50
Total	**24**	**100**	**16**	**100**	**20**	**100**	**20**	**100**
	χ^2=5.293; ***p*= 0.021**				χ^2=3.956; ***p*=0.046**			
Grade								
Low	21	87.5	9	56.25	18	90	12	60
High	3	12.5	7	43.75	2	10	8	40
Total	**24**	**100**	**16**	**100**	**20**	**100**	**20**	**100**
	χ^2=2.222; ***p*= 0.136**				χ^2=4.800 ***p*= 0.028**			

DISCUSSIONS

From recent study, it has been found that Ninety-five percent of new cases and 97% of breast cancer deaths occurred in women 40 years of age and older. During 2004-2008, the median age at the time of breast cancer diagnosis was 61 years.[3]However in our study of 40 patients, the age ranged between 35 to 65years with median age at the time of diagnosis was 45 years. Our finding is parallel to that of another Indian study where average age of Breast cancer in Asian women is in their forties[8] and the same in USA and Europe, it is in their sixties. The most consistent symptom was breast lump present in all 40 presents (100%), similar as reported in other Indian studies [9,10] followed by pain in 32.5% of patients, lump axilla and nipple discharge in 5% patients each. Majority of the patients (57.5%) had their symptoms duration between 6-12 months. Whereas, Sandhu et al (2010) reported 70.4% of patients to present within 6 months of

onset of symptoms[11]. Left side laterality was more common, seen in 22 out of 40 patients (55.0%), in accordance with other studies [4,12]. The possible explanation given in mentioned studies is more bulk of left breast as compared to right.

In this study, MVD was determined in 40 patients, which ranged from 5 to 19 with mean of 11.45 ± 3.94. Where as LVD ranged from 7 to 35 with mean of 17.92±7.38. In another study, Jan Rykalaet al in 2011[13] used CD34 antibody as marker to quantify angiogenesis in breast cancer and correlate with other clinic opatho logical prognostic variables, where the MVD determined in 54 patients by, ranged from 0.0 to 110.0 with mean of 26.Between-study variations could due to patient selection criteria, use of different antibodies, techniques to stain and count micro vessels.

Result of our study shows that high LVD correlated significantly with higher grade of tumor (χ^2=4.800; p= 0.028). However, there is no significant association of MVD with grade (χ^2=2.222; p= 0.136). Schopmann et al (2004) showed that high LVD was associated with a higher differentiation grade tumor[14]. It led to speculation that fast growing tumors produced more growth factors and offer a bigger clonal variety of tumor cells capable of involving lymphatic vessels compared with well differentiated slow growing tumors. Kato et al (2003)[15] had also showed similar results with respect to association of grade with LVD (p=0.0434).

Tumor angiogenesis has been reported to have an important role in the metastasis of breast cancer and tumor blood vessel density has been reported to be associated with axillary lymph node metastasis. In our study the presence of lymph node metastasis was significantly correlated with MVD (r = 0.447, p= 0.004) and LVD (r = 0.397, p = .011) score. This significant correlation between MVD with lymph node involvement could be explained as there is an elevated expression of angiogentic markers like VEGF, TIMP-1 and ICAM-1 in patients with lymph node metastasis [13] and LVD with lymph node metastasis through lymphangiogenesis-induced increase of the lymphatic vessel. However there exist no correlation between histopathologically total lymph nodes count and LVD (r = 0.966, p= -.007) and no significant correlation with MVD (r= 0.251, p=0.118). Some other studies also found significant correlation of CD-31 detected blood microvessel density with lymph node metastasis[16,17,18]

ER negative tumors shows show more malignant behavior than ER positive tumors and it is therefore possible that the biological behavior of breast cancer associated with MVD might be affected by ER status. We found, MVD is significantly correlating with ER negative status (p<0.014). However, there is no correlation between MVD or LVD with PR and HER-2 neu receptor status. Y Ogawa et al (1995) found MVD of ER negative tumors was higher than that of ER positive tumors[18]. Rabab A et al(2009) found significant association of high LVD with ER negative tumors[19].

Limitation

This is a small study and the choice of different endothelial markers and the region of tumor representation from specimen used for IHC staining may modify the conclusion. A single tortuous vessel in block when cut tangentially may present as multiple microvessel lumens in slide thus leading to false exponential increase in MVD or LVD.

CONCLUSIONS

To conclude, this study has found a statistically significant correlation between angiogenesis and lymphangiogenesis assed by MVD and LVD which was determined quantitatively by immunohistochemistry technique. Moreover they also correlate significantly to many of the well know established prognostic factors of breast cancer like tumour size stage, grade, vascular invasion, and positive lymph nodes. So this study result proves that angiogenetic and lymphangiogenetic

factors play an important role in tumour growth, invasion and metastasis. So they can be used as prognostic markers of breast cancer.

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